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DOI:
10.1016/j.fsi.2009.02.020

Document Version
Peer reviewed version

Citation for published version (Harvard):
Osuna-Jimenez, I, Williams, T, Prieto-Alamo, MJ, Abril, N, Chipman, J & Pueyo, C 2009, 'Immune- and stress-related transcriptomic responses of Solea senegalensis stimulated with lipopolysaccharide and copper sulphate using heterologous cDNA microarrays', *Fish & Shellfish Immunology*, vol. 26, no. 5, pp. 699-706. https://doi.org/10.1016/j.fsi.2009.02.020

Fish & Shellfish Immunology 26 (2009) 699–706

Contents lists available at ScienceDirect

Fish & Shellfish Immunology

journal homepage: www.elsevier.com/locate/fsi

Immune- and stress-related transcriptomic responses of *Solea senegalensis* stimulated with lipopolysaccharide and copper sulphate using heterologous cDNA microarrays

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ARTICLE INFO

Article history:
Received 27 November 2008
Received in revised form
9 February 2009
Accepted 21 February 2009
Available online 3 March 2009

Keywords:
Senegalese sole
Hepatic mRNA expression profiling
Heterologous microarray
qRT-PCR
LPS
$CuSO_4$
Aquaculture

ABSTRACT

The sole, *Solea senegalensis*, is a common flatfish of Atlantic and Mediterranean waters with a high potential for aquaculture. However, its cultivation is hampered by high sensitivity to different stresses and several infectious diseases. Improving protection from pathogens and stressors is thus a key step in reaching a standardized production. Fish were exposed to lipopolysaccharide (LPS), a mimetic of bacterial infections, and copper sulphate ($CuSO_4$), used in aquaculture to control algae and outbreaks of infectious diseases. We employed a European flounder cDNA microarray to determine the transcriptomic responses of Senegalese sole to these exposures. Microarray analyses showed that many genes were altered in expression following both LPS and copper treatments in comparison to vehicle controls. Gene ontology analysis highlighted copper-specific induction of genes related to cellular adhesion and cell signalling, LPS-specific induction of genes related to the immune response, and a common induction of genes related to unfolded protein binding, intracellular transport/secretion and proteasome. Additionally transcripts for glutathione-*S*-transferases were down-regulated by LPS, and those for digestive enzymes were down-regulated by both treatments. We selected nine changing genes for absolute quantification of transcript copy numbers by real-time RT-PCR to validate microarray differential expression and to assess inter-individual variability in individual fishes. The quantitative RT-PCR data correlated highly with the microarray results. Overall, data reported provide novel insights into the molecular pathways that could mediate the immune and heavy metal stress responses in Senegalese sole and thus might have biotechnological applications in the culture of this important fish species.

1. Introduction

The sole, *Solea senegalensis*, is a common high-value flatfish that has become a priority species for the diversification of aquaculture in Southern Europe. However, its cultivation is hampered by its high sensitivity to different stresses and to several infectious diseases that can cause large mortalities [1]. Consequently, there is a need to identify candidate genes as potential molecular biomarkers responsive to stress, infections and pollutants, with a view to improving productivity, management and fish welfare in aquaculture.

The lipopolysaccharide (LPS) fraction of Gram-negative bacteria is commonly used as a mimetic of bacterial infections. LPS is a complex molecule composed of a polysaccharide chain and a toxic lipid moiety responsible for its immunostimulatory characteristics [2]. LPS loading of the blood can be raised not only through bacterial infection but also through dysfunction of the gut wall such as might occur from chemical exposure (e.g. ethanol) in various animal models [3]. Another stressor (metal exposure) of interest is copper sulphate ($CuSO_4$) due to its use in aquaculture as bacteriocide and algaecide [4].

This study aimed to characterize the transcriptomic responses of *S. senegalensis* to LPS and $CuSO_4$. Analyses focussed on the liver since it is the main organ of xenobiotic detoxification and where

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doi:10.1016/j.fsi.2009.02.020

the majority of plasma proteins involved in the innate immune response is produced, involving the action of Kupffer cells. Indeed the hepatotoxicity of LPS in mammalian species has been shown to be, at least in part, mediated by cytokine release from Kupffer cells [5]. DNA-based microarrays represent a powerful high-throughput analytic technology for examining multigene expression patterns. The use of microarrays manufactured from transcripts of one species to probe gene expression in another, related, species, eliminates the need to fabricate a new microarray platform for every new species of interest [6]. Very limited genomic information was available for *S. senegalensis* until the recent development of a microarray resource for this species [7]. However as this was not available at the time of the study we used a European flounder (*Platichthys flesus*) cDNA microarray [8]. This microarray has been found to be effective for hepatic gene expression analysis of many species of the order Pleuronectiformes [9]. Additionally we performed gene ontology analyses to determine functionally related groups of genes that were altered by the treatments. Subsequently we quantified the transcript copy numbers of nine selected genes by real-time polymerase chain reaction (qRT-PCR) to validate the microarray responses and to further quantify the transcriptional profiles of these genes in individual fish.

2. Materials and methods

2.1. Treatments

Fish (65 ± 13 g) of mixed sex were from domesticated broodstocks at the "Centros IFAPA *Agua del Pino* and *El Toruño*" (Andalucía, Spain). Larvae were fed on rotifers and *Artemia nauplii* and weaned onto an artificial diet at 1–2 months post-hatch, which was then substituted by commercial *Solea* diet (Skretting, LE-ELITE). Prior to challenges, soles were transferred and maintained (2 weeks) for adaptation in 100 L flat-bottom tanks (20 animals per tank), in an open circulation system (300% water renewal/day, 20 ± 1 °C temperature and $35 \pm 1‰$ salinity) with 16L:8D photoperiod and a light intensity of 600–800 lux, and fasted 24 h prior to and during the experiments. Fish were treated by a single intraperitoneal (IP) injection with LPS serotype 0111:B4 (25 mg kg^{-1}) or $CuSO_4$ (2 mg kg^{-1}) in 100 μl phosphate-buffered saline (PBS). The expression of fish immune-relevant genes is routinely investigated following IP injection with pathogenic bacteria or LPS. Therefore, it has recently been shown that IP-injected LPS at similar dose (30 mg kg^{-1}) to that used in the current study produces a significant transcriptional response of the c-type lysozyme gene in Senegalese sole [10]. To simplify the exposure experiments and to reduce the number of animals to be studied, copper contamination was also performed by IP injection. The copper dose (2 mg kg^{-1}) was chosen as it has been seen to induce oxidative stress when IP injected into the sea bass (*Dicentrarchus labrax*) at the times used in this experiment [11]. Individuals injected with 100 μl PBS served as vehicle controls of all LPS- and $CuSO_4$-treated fish. Neither mortality nor phenotypic effects were observed in any of the fish. Soles were sacrificed by immersion in 50 ppm tricaine methanesulphonate (MS222) and livers were immediately removed and frozen in liquid nitrogen. Two treatments were carried out, in which fish were sampled at 6 and 24 h or at 3, 12 and 24 h post-injection. In the first, the livers from at least 10 individuals/experimental condition were pooled for microarray analyses and qRT-PCR validation. In the second experiment, liver samples from eight treated and eight control fish were individually analysed by qRT-PCR. Total RNA was extracted as detailed in [12]. RNA sample quality was checked electrophoretically, and spectrophotometrically quantified.

2.2. Microarray experiments

The European flounder GENIPOL microarray has been described previously [8] and represents 3336 unique expressed sequence tag (EST) clusters. The majority of the cDNA clones arrayed were derived from a hepatic cDNA library of flounders treated with a variety of model toxicants. Briefly, total RNA derived from pooled livers was reverse-transcribed with Superscript II (Invitrogen) using oligo-dT primers (Alta Bioscience) before repurification (Qiagen). cDNA was labelled with Cy5-dCTP or Cy3-dCTP (Amersham) using Klenow polymerase (Invitrogen) with random primers. Labelled cDNA was purified (Qiagen), and fluorophore incorporation measured by spectrophotometry. Arrays were hybridized with the labelled cDNA samples derived from pooled samples from each of the experimental conditions (PBS-, LPS- or $CuSO_4$-injected soles for 6 or 24 h), and cDNA from a common reference consisting of a mixture of hepatic RNA from PBS-injected fish for 6 and 24 h. Three forward- and three reverse-labelled technical replicate hybridizations (dye-swap design) were carried out in a random order for each sample. Cy5 and Cy3 dye-labelled cDNA (60 pmol incorporated of each) were hybridized to one microarray slide. Hybridizations were carried out for 18 h, before stringent washing and scanning (Axon 4000B with Genepix software, Molecular Devices). The data used in analyses consisted of local background-subtracted measurements. The generated data set has been submitted to ArrayExpress at EMBL–EBI using maxdLoad2 software and has been assigned the accession number *E-MAXD-47*.

2.3. Microarray data analysis

The Genespring v7.2 software (Agilent) was used to analyse microarray data. Clones corresponding to the same gene (i.e. contigs) were considered as replicate spots. To normalize, data were Lowess transformed. Each microarray in a treatment group was normalized to the mean of the relevant control group. Low intensity and highly variable spots were removed by filtering. Spots with an intensity <76 (calculated using the cross-gene error model component of Genespring) in the control channel were discarded as were genes showing a standard deviation of >1.4 between replicate spots in four or more conditions. Of genes represented on the array, 86% passed these thresholds. Statistically significant differences were determined by parametric Welch *t*-test between test and control groups. The *P*-value cutoff was 0.05, and the Benjamini and Hochberg multiple-testing correction [13] was used, resulting in a 0.05 false discovery rate (FDR). Similarity between sample groups was assessed by Spearman clustering within Genespring. Blast2GO [14] was used to compare the representation of GO terms in the lists of statistically significantly differentially expressed genes compared to the whole gene set by employing Fisher's Exact Test with a multiple-testing correction under the Gossip package [15]. The cutoff threshold for statistical significance was set at a false discovery rate (FDR) <0.05.

2.4. Primers

The *S. senegalensis* sequence of GAPDH1 gene was from GenBank database (*AB300322*). Sole sequences of PSMD3, HMGB2, DDIT4L, AGT, and NARS were from the dbEST of GenBank (*FF682487*, *FF682560*, *FF682562*, *FF682392* and *FF682551*, respectively). Degenerate primers (Supplementary file 1) to amplify HAMP, TNFAIP9 and GP96 sole genes were designed by alignment of conserved regions of available sequences from at least three fish species, using Oligo 6.1.1/98 software (Molecular Biology Insights).

The PCR products were sequenced and are available as GenBank accession numbers *FJ263548*, *FJ263550* and *FJ263549*, respectively. The identity of the sequences was confirmed using tBLASTx algorithm on the BLAST server at the NCBI databank. Primers for qRT-PCR (Supplementary file 2) of the nine selected transcripts were designed as detailed in [12]. All primer pairs produced amplicons of the predicted size. All PCR products were further verified by nucleotide sequencing.

Table 1
Selected genes statistically significantly (FDR < 0.05) differentially expressed during copper and/or LPS treatments.

Systematic	Putative Identity	GenBank	Copper 6	Copper 24	LPS 6	LPS 24
Cell junction						
PfIL291D09	Claudin 26	*DV569360*	1.341	0.899	0.805	1.033
PfIL305H02	G-protein-coupled receptor kinase-interactor 2 isoform 1	*EC379374*	1.540	0.772	0.877	0.926
Immune response						
Contig338	**Hepcidin precursor (HAMP)**	*DV566288*	1.216	1.045	6.939	2.361
Contig342	**TNFα-induced adipose-related protein (TNFAIP9)**	*DV568017*	1.170	1.489	7.452	4.774
Contig59	Interleukin 8 precursor	*DV565292*	1.198	1.498	1.758	1.941
Contig564	Similar to interleukin 25	*DV566068*	0.807	1.138	0.990	1.822
Contig44	Chemotaxin (LECT2)	*DV565320*	0.831	1.181	1.316	2.341
PfIL254F08	Nf-kappa B activator (TANK)	*DV567872*	1.219	0.975	2.742	1.257
PfIL284G03	B-cell leukemia/lymphoma 3 (BCL-3)	*EC379043*	1.112	0.940	2.817	1.060
PfIL228A07	Complement component C3	*EC378166*	0.817	1.048	1.067	1.452
PfIL250D12	Complement component C7	*DV567673*	1.948	2.088	2.638	3.339
Glutathione-S-transferases						
Contig362	Glutathione-*S*-transferase (GST-A)	*DV565329*	0.866	1.078	0.941	0.429
PfIL238F01	Glutathione-*S*-transferase	*EC378322*	0.907	0.894	0.871	0.521
PfIL260C08	Glutathione-*S*-transferase, theta 3	*DV568150*	0.891	0.932	0.996	0.688
PfIL304H03	Microsomal glutathione-*S*-transferase 1	*EC379360*	0.855	1.393	0.979	0.610
Contig981	Microsomal glutathione-*S*-transferase 3	*AJ605273*	0.863	1.108	0.983	0.725
Digestive enzymes						
Contig914	Trypsinogen 2 precursor	*DV568965*	0.420	0.652	0.626	0.191
Contig633	Chymotrypsin B precursor	*DV567597*	0.495	0.773	0.721	0.316
PfIL215C03	Elastase 4 precursor	*DV566319*	0.482	0.751	0.745	0.298
PfIL007A06	Carboxypeptidase B	*DV565430*	0.495	0.825	0.715	0.397
Contig648	Carboxypeptidase A1	*DV565300*	0.411	0.743	0.648	0.230
Unfolded protein binding						
PfIL255E12	**Heat shock protein gp96 (GP96)**	*DV567919*	0.947	1.999	1.258	3.913
Contig960	Peptidyl-prolyl isomerase	*DV565676*	0.999	1.704	1.093	2.641
Contig159	15 kDa selenoprotein precursor (SEP15)	*DV568180*	0.892	1.565	1.097	2.269
PfIL273G12	Prostaglandin E synthase 3, telomerase binding protein, p23	*DV568689*	1.003	2.202	1.143	1.851
PfIL294H06	Heat shock 70 kDa protein 4	*DV569489*	1.086	2.042	1.022	1.906
Intracellular transport/secretion						
PfIL222F11	Coatomer protein complex, subunit beta 1	*DV566527*	1.044	1.653	1.492	2.136
PfIL314E01	Coatomer epsilon subunit	*DV570223*	0.960	1.934	1.349	2.272
PfIL294D10	ADP-ribosylation factor 5 (ARF5)	*DV569467*	1.105	2.115	1.680	2.530
Contig307	Transmembrane emp24 protein transport domain 7 (TMED7)	*DV567466*	1.040	1.507	1.230	1.893
PfIL254H09	Transmembrane trafficking protein, TMP21	*DV567887*	0.961	1.642	1.458	2.421
PfIL230G07	SEC22, vesicle trafficking protein-like 1B	*DV566831*	0.864	1.750	1.149	2.162
Contig1095	Transport protein Sec61 beta-subunit	*AJ543354*	0.988	1.532	1.260	2.171
Proteasome						
Contig714	Proteasome 26S ATPase subunit 5, MSUG1/RPT6/S8	*DV568919*	1.101	1.997	1.371	2.337
PfIL266F02	**Proteasome 26S non-ATPase subunit 3/RPN3 (PSMD3)**	*DV568418*	0.916	1.507	1.028	1.695
PfIL308H05	Proteasome 26S, non-ATPase regulatory subunit 6/RPN7	*DV570012*	0.993	1.412	1.014	1.502
Contig443	Proteasome 26S subunit, non-ATPase, 12/RPN12	*DV565642*	1.044	1.405	1.155	1.348
PfIL258H08	Proteasome alpha 1 subunit isoform 2	*DV568071*	0.977	1.555	1.144	2.106
Contig388	Proteasome subunit, alpha type, 6	*DV570235*	1.019	1.311	1.117	1.551
Contig168	Proteasome subunit, alpha type, 7	*DV566606*	0.976	1.725	1.181	2.169
PfIL273D04	Proteasome beta-subunit C5 (proteasome subunit, beta type, 1)	*DV568672*	0.957	1.782	1.241	2.381
Contig581	Proteasome subunit, beta type, 3	*DV565424*	0.970	1.641	1.277	2.013
PfIL226E10	Proteasome subunit, beta type, 5	*DV566673*	0.955	1.373	0.987	1.772
Contig341	Proteasome delta/subunit, beta type, 6	*DV567140*	0.933	1.225	0.946	1.587
Contig250	Proteasome subunit N3 (proteasome subunit, beta type, 7)	*DV566865*	0.927	1.169	0.965	1.444
Contig601	Proteasome subunit, beta type, 8	*DV566832*	0.921	0.982	1.061	1.230
PfIL255H08	Proteasome activator subunit 2/PA28β	*DV567935*	1.035	1.015	1.255	1.416
Miscellanea						
Contig422	**Glyceraldehyde-3-phosphate dehydrogenase (GAPDH1)**	*AJ305222*	0.870	0.814	0.772	0.643
Contig330	**Asparaginyl-tRNA synthetase (NARS)**	*DV567690*	1.506	4.451	1.410	3.154
Contig647	**Angiotensinogen precursor (AGT)**	*DV565437*	1.631	2.516	2.002	2.243
Contig310	**NHP2 non-histone chromosome protein 2-like 1 (HMGB2)**	*DV566614*	1.007	2.223	1.097	1.665
Contig500	**DNA-damage-inducible transcript 4-like (DDIT4L)**	*AJ580016*	1.093	1.988	1.060	1.082

Representative GenBank accession numbers are shown for contigs.
Expression changes greater than 1.5-fold in comparison with controls are highlighted in grey.
Selected genes for qRT-PCR quantification are highlighted in bold.

2.5. qRT-PCR

Absolute quantification of mRNA levels by qRT-PCR was carried out as detailed in [12]. Briefly, cDNA was generated from 2 μg of RNA. Real-time PCR reactions were performed in quadruplicate by using 50 ng of cDNA. No primer dimers were detected. All primers designed for absolute quantification of selected targets showed optimal (~100%) PCR efficiencies in the range of 20–2×10^5 pg of total RNA input with high linearity ($r > 0.99$). An absolute calibration curve was constructed with an external standard in the range of 10^2–10^9 RNA molecules. The number of transcript molecules was calculated from the linear regression of the standard curve.

3. Results

3.1. Responses to treatments analysed by microarrays

Statistical analyses (FDR < 0.05) showed that 405 genes were differentially expressed with copper treatment at 6 h, 468 with copper at 24 h, 271 with LPS at 6 h and 664 with LPS at 24 h, in comparison between pools of test and PBS control samples, considering the technical variation from six microarrays per group (Supplementary file 3). To facilitate the discussion, the microarray data for a selected group of genes are given in Table 1. The GO terms that were over-represented amongst lists of differentially expressed genes are shown in Supplementary file 4.

Spearman cluster analysis was used to show the responses of those genes that were identifiable with known proteins and were statistically significantly differentially expressed. All sample groups segregated well, except for the PBS controls for which 6 and 24 h samples could not be distinguished, implying no overall alteration in gene expression in the controls over this time period (Fig. 1).

3.2. Absolute transcriptional profiling by qRT-PCR

To verify that candidate genes identified in the heterologous microarrays were indeed differentially expressed, we quantified the transcript copy numbers of nine selected genes. These genes were chosen because their reported functions made them interesting candidates, they represent a variety of functional classes and appeared either up- or down-regulated in the microarray experiments (Table 1).

Validation by qRT-PCR was performed first with the same pooled samples employed in the microarray experiments. The results are shown in Supplementary file 5, and they are presented as conventional fold variations in Table 2 in order to compare both methodologies. While microarray and qRT-PCR data corresponded qualitatively, the absolute quantification by qRT-PCR was more sensitive as, in all cases, the magnitude of the changes detected with this technique was greater. This was not unexpected as cDNA arrays may be less specific than qRT-PCR, with non-specific binding of transcripts, especially those from different members of the same gene families. This effect may be exacerbated in cross-species cDNA arrays where the probes are unlikely to have 100% nucleotide identity with their targets. Nevertheless microarray and qRT-PCR data were highly correlated ($R^2 = 0.931$) where the RT-PCR fold change was less than 14 (Supplementary file 6).

To analyse in depth the transcriptional profile of selected genes and determine the effects of inter-individual variability, a second exposure to the same concentrations of LPS and $CuSO_4$ was carried out for 3, 12 and 24 h with eight individuals per experimental condition. Data at the individual level (Fig. 2) confirmed the results obtained in samples from mixtures of individuals (Table 2). Different expression patterns were distinguished, regarding both the stressor specificity and the time-course response. Hence, TNFAIP9 and HAMP responded specifically to LPS, their maximal inductions occurred at the shortest exposure time (3 h in this second experiment). GP96 and NARS, with greater response to LPS than to copper, showed their maximal induction by LPS at 12 h and a relatively early response to copper, this response was not seen in pooled samples. PSMD3, AGT, HMGB2 and DDIT4L were transcripts that responded non-specifically to both treatments. The greatest discrepancy between pooled and individual samples was observed with DDIT4L. This may be due to the presence in the pooled samples ($CuSO_4$, 24 h) of one or more animals with abnormally high levels of this transcript, which had the lowest overall basal level (Supplementary file 5). GAPDH, frequently used as a housekeeping marker in transcript quantifications, was repressed in response to LPS, which correlated well with the microarray data. Additionally, we noted that a high number (~50%) of genes isolated by suppressive subtractive hybridization experiments [16] were also identified as differentially expressed by microarray analysis, providing further confidence in the data.

4. Discussion

4.1. Response to copper treatment

Copper is a trace element essential for cellular metabolism but may become extremely toxic for aquatic animals at high concentration. The impact of copper on the aquatic environment is complex and depends on the physicochemical characteristics of water [17]. Interestingly, only the GO term cell junction was statistically significantly over-represented amongst induced genes after copper treatment for 6 h. This term grouped genes related to cellular adhesion, such as claudins which are integral transmembrane tight junction proteins involved in regulating paracellular permeability [18], and those related to cell signalling as G-protein-coupled receptor kinase-interactor 2 involved in membrane trafficking between the plasma membrane and the recycling endosome [19]. This finding is in agreement with the ability of copper to alter tight junction permeability in human intestinal mucosa [20]. Of note also

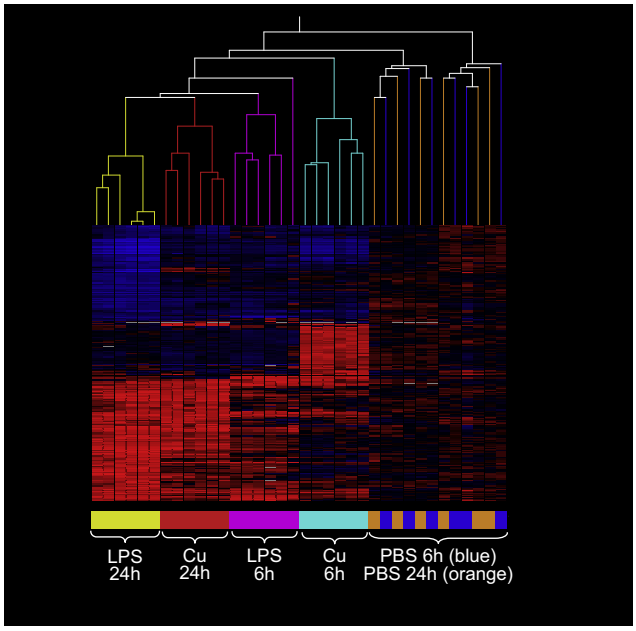

Fig. 1. Spearman clustering of genes statistically significantly changed (≥1.5-fold) in expression between treated and control fish. Genes are shown vertically, samples horizontally, red indicates induction, and blue indicates repression. The maximum colour intensity was set to 2-fold change, grey indicates no data.

is the observation that many of the statistically significantly up-regulated genes at 6 h after copper challenge were not identifiable by homology with known proteins, providing opportunities for discovery of novel short-term responses to this metal.

4.2. Response to LPS treatment

As shown by GO analyses (Supplementary file 4) genes related to the immune response were induced specifically by LPS, in accordance with its immunostimulatory properties [2]. These included hepcidin (HAMP), an antimicrobial peptide and a component of the innate immune system that plays an important role in defence against invading pathogens. Numerous teleost HAMP sequences have been published, and several reports demonstrate an up-regulation of gene expression after treatment with LPS, bacterins or live bacteria ([21] and references herein). Our results support this function for sole HAMP, given its high and early up-regulation by LPS (not by copper treatment), and the corroboration of these results by qRT-PCR at the individual level. TNFα-induced adipose-related protein (TNFAIP9) was also found by qRT-PCR to be quickly, highly and specifically up-regulated by LPS, therefore confirming the microarray results. Mammalian TNFAIP9 (also named TIARP and STEAP4) is induced by TNF-alpha as a protective anti-inflammatory factor [22]. Murine TNFAIP9 has recently been characterized as displaying metalloreductase activity *in vitro*, capable of facilitating cellular uptake of iron and copper [23]. Whereas the biological functions of fish TNFAIP9 are presently unknown, its pattern of expression following LPS and copper treatments suggests that sole TNFAIP9 is regulated more by the inflammatory response than through copper homeostasis.

Other immune-related genes up-regulated by LPS include cytokines, chemotaxins, NF-κB activator (TANK) and complement proteins. Cytokines are major signalling molecules involved in immunity and include tumour necrosis factors (TNFs), interleukins, chemokines and interferons. Chemokines are involved in chemotaxis of immune cells towards the site of wound or infections. Interleukin 8 is a CXC chemokine [24]. Interleukin 25, recently identified as a member of the interleukin 17 family of cytokines, has potent pro-inflammatory effects and induces the production of chemokines such as interleukin 8 [25]. Leucocyte cell-derived chemotaxin 2 (LECT2), a multifunctional protein involved in cell growth, differentiation, damage/repair and autoimmune response, was initially isolated as a chemotactic factor for human neutrophils. Its expression was induced in different fish species upon bacterial infection [26]. TANK is a signal mediator of NF-κB activation. Mammalian TANK seems to be an important component in innate immune responses by playing a critical role in LPS-mediated type I interferon induction [27]. The deleterious effects of prolonged and elevated levels of TNFα require a stringent control over its expression. BCL-3 negatively regulates the transcription of the pro-inflammatory cytokine TNFα, by blocking the NF-κB p50 ubiquitation. Hence, BCL-3-deficient mice are unable to control the LPS-induced inflammatory responses [28]. Overall the expression of inflammation-related genes in liver tissue is likely to involve a complex interaction between Kupffer and parenchymal cells, given that it is well-recognized in mammals that cytokine release from Kupffer cells can alter signalling and metabolic functions in neighbouring hepatocytes [29]. Thus various changes in gene expression may occur not only in hepatocytes but also in the Kupffer cells and additional changes in hepatocytes may be secondary to the Kupffer cell modifications.

Glutathione-*S*-transferases (GSTs) were more prevalent in the list of transcripts down-regulated at 24 h after LPS treatment. GSTs are phase II enzymes involved in detoxification and bioactivation of xenobiotics and endogenous electrophilic compounds. Many studies have revealed multiple interactions between biotransformation and immune systems in vertebrates. The capacity of bacterial infection and LPS to down-regulate biotransformation activities such as GSTs has been shown in a number of fish species [30]. In mammals, this down-regulation has been linked to a mutual inhibitory interaction between the aryl hydrocarbon receptor and the NF-κB signalling pathways [31]. Understanding of possible interactions between fish immune and biotransformation systems should improve fish health monitoring which is crucial in aquaculture. GAPDH expression was also specifically down-regulated by LPS. This result was verified by qRT-PCR and it is in agreement with previous data on rainbow trout [32]. In mammals, it is now widely accepted that GAPDH is not simply a glycolytic enzyme, but a multifunctional protein with defined functions in numerous cellular processes, including a number of immune and disease-relevant pathways [33].

Table 2
Comparison of relative gene expression levels determined by microarray and qRT-PCR.

Gene	Time (h)	Cu		LPS	
		MICROARRAY Fold variation	qRT-PCR Fold variation[a]	MICROARRAY Fold variation	qRT-PCR Fold variation
TNFAIP9	6	1.17	1.38	7.45	25.41
	24	1.49	2.00	4.77	10.16
HAMP	6	1.22	1.69	6.94	61.15
	24	1.05	1.00	2.36	14.17
GP96	6	0.95	0.73	1.26	1.76
	24	2.00	2.54	3.91	6.58
NARS	6	1.51	2.33	1.41	2.33
	24	4.45	8.00	3.15	5.00
PSMD3	6	0.92	1.00	1.03	1.09
	24	1.51	2.40	1.70	2.30
AGT	6	1.63	2.64	2.00	3.79
	24	2.52	3.48	2.24	3.08
HMGB2	6	1.01	1.00	1.10	1.32
	24	2.22	1.60	1.67	2.18
DDIT4L	6	1.09	2.00	1.06	2.00
	24	1.99	42.00	1.08	3.00
GAPDH	6	0.87	0.77	0.77	0.93
	24	0.81	0.88	0.64	0.79

[a] Absolute transcript levels determined by qRT-PCR are shown in Supplementary file 2.

4.3. Common responses to the two treatments

Analyses of transcriptional patterns and GO terms highlighted that copper and LPS treatments resulted in a common response for genes corresponding to four groups of GO categories, i.e. down-regulation of digestive enzymes, and up-regulation of unfolded protein binding, intracellular transport and secretion, and proteasomal proteins. Digestive enzymes such as trypsin, chymotrypsin, elastase, and carboxypeptidase A and B were down-regulated in response to both treatments, although fish were fasted during the experiment. A similar down-regulation has been recently reported in striped sea bream on exposure to cadmium [34] and we echo the hypothesis that this might be a consequence of a general stress caused by the treatments.

Extensive studies in mammalian cells have shown that disruption of endoplasmic reticulum (ER) homeostasis leads to the accumulation of unfolded proteins in the ER and activation of the unfolded protein response (UPR). To increase the folding capacity of the ER, the synthesis of ER molecular chaperones (including GP96) and foldases (including peptidyl-prolyl isomerase and selenocysteine-containing oxidoreductase Sep15) is increased (reviewed in [35]). This adaptive response allows cells to survive usually

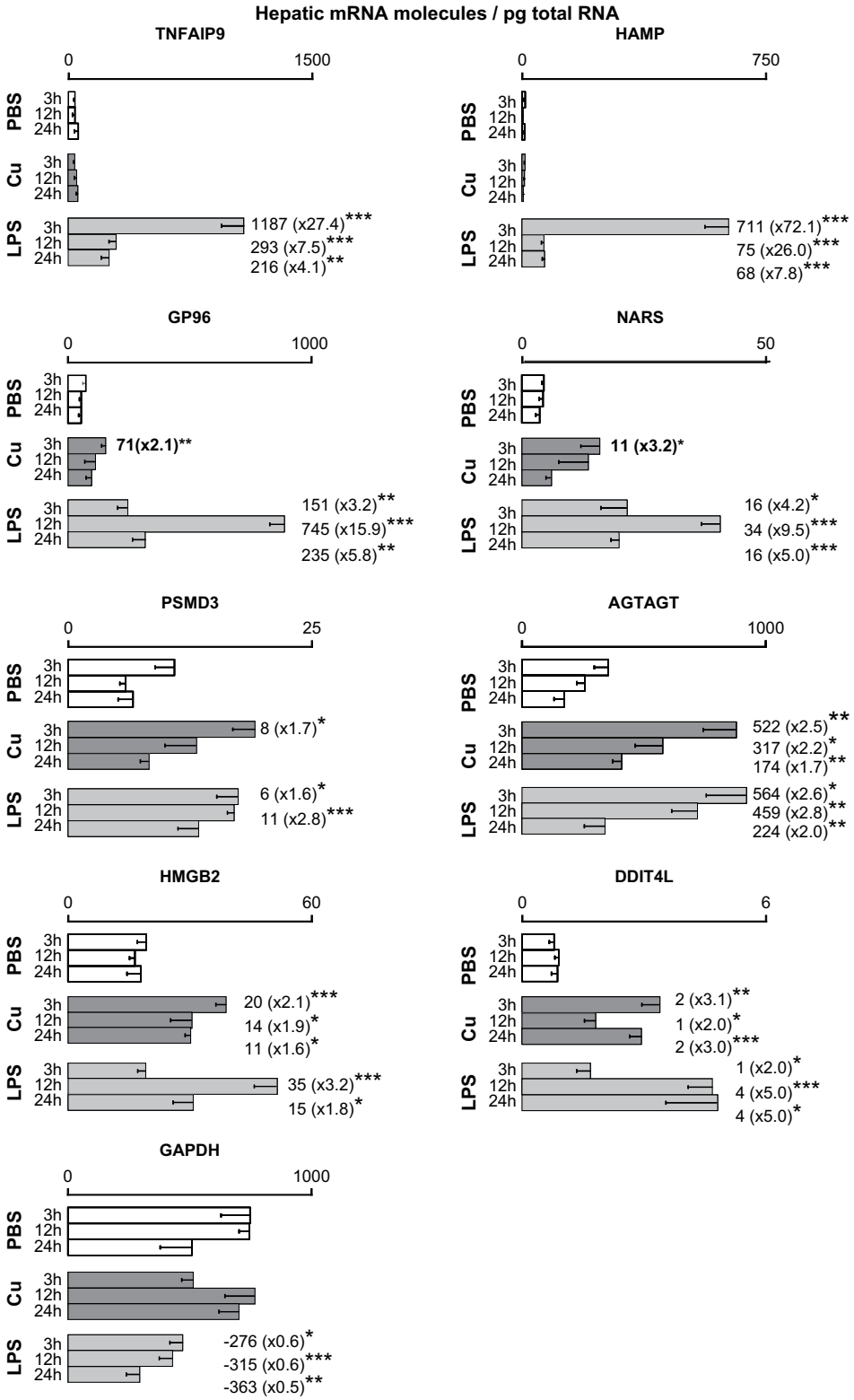

Fig. 2. Differences in transcript levels in response to $CuSO_4$ or LPS. Data are the means ± SEM of transcript molecules/pg of total RNA from eight fish in each group. Comparisons were made by Student's *t*-test. Statistical significance is expressed as: $^{***}P < 0.001$, $^{**}P < 0.01$, and $^{*}P < 0.05$. The significant increases or decreases in mRNA copy numbers and the conventional fold variations (in parentheses) are given for comparison.

reversible environmental stresses. However, if stress conditions are not relieved, apoptotic pathways are activated. The small chaperone p23 plays a role in modulating ER stress-induced apoptosis [36]. Our microarray data indicate that sole transcripts coding for this group of proteins are up-regulated in response to both copper and LPS, implying that protein folding in the ER has been perturbed. Most studies on fish chaperones have focused on the small heat shock proteins (major cytosolic chaperone classes), like Hsp70s. As

in other organisms, elevated levels of HSPs are induced in fish tissues in response to different kinds of stress conditions, such as bacterial infections or exposure to heavy metals [37], to prevent misfolded proteins from forming aggregates that can become cytotoxic. In accord with this, sole Hsp70 mRNA was induced in response to the two treatments. HSPs are also well-known to be involved in the immune response in mammals. GP96 (a Hsp90 homolog), localised in the ER, is endowed with crucial immunological functions for priming innate and adaptive immunity [38]. Quantification by qRT-PCR indicated that sole GP96 mRNA was more highly induced following LPS challenge than following copper challenge.

The ER is essentially a folding factory, from which correctly folded proteins are transported towards their final destination. Both treatments increased the levels of transcripts coding for proteins involved in the biogenesis, budding and traffic of COPI- and COPII-coated vesicles that mediate ER/Golgi protein transport within the secretory pathways [39,40]. These transcripts included those encoding coatomer beta and epsilon subunits, ADP-ribosylation factor 5 (ARF5), several members of the p24 protein family (TMED7, TMP21) and the SNARE protein Sec22. Interestingly, both treatments increased the mRNA levels of one major subunit of the ER translocation channel (Sec61), through which polypeptides traverse the ER membrane [41]. Our results are in agreement with previously reported changes in murine SNARE protein levels in response to LPS, accommodating the rapid onset of cytokine secretion and membrane traffic associated with the phenotypic changes of immune activation [42].

The proteasome is a large cytosolic protease complex that degrades unneeded or damaged proteins. Additionally, proteins that never fold into a transport-competent state are retrotranslocated to the cytosol (in a process called ER-associated degradation) to be degraded by the proteasome. A recent microarray study has shown that key signalling pathways induced by LPS are regulated by the proteasome [43]. To this end, LPS binds directly to selected proteasome subunits (like N3), and consequently activates proteasomal activity [44]. It has been established that oxidative stress up-regulates the ubiquitin proteasome pathway [45]. In agreement with these previous results in mammalian cells, here we show that 14 transcripts coding for different components of both the 20S-protease and 19S-regulatory proteasome complexes were induced following LPS and copper treatments. qRT-PCR, complementing the microarray analysis, confirmed the up-regulation, by both LPS and copper treatments, of PSMD3 mRNA, which codes for an essential non-ATPase regulatory subunit at the proteasome lid.

Four other transcripts, selected for qRT-PCR quantification, were also found to be up-regulated by the two treatments. Aminoacyl-tRNA synthetases, such as NARS, play a fundamental role in protein synthesis and display additional functions. Human NARS is thus a potent pro-inflammatory chemokine [46]. Up-regulation of sole NARS mRNA, with larger response to LPS than to copper, represents an interesting starting point to unravel novel secondary activities for piscine aminoacyl-tRNA synthetases. Angiotensinogen (AGT) is thought to be an acute phase protein. Up-regulation of sole AGT mRNA levels by LPS challenge is in agreement with previous data in mammals and in ayu fish (*Plecoglossus altivelis*) [47,48]. Copper up-regulation of AGT mRNA levels is in accord with the well-recognized role of angiotensinogen in the regulation of blood pressure and fluid homeostasis and with the well-known effect of copper on the osmoregulatory and ionoregulatory physiology of fish [49]. HMGs are well-conserved eukaryotic non-histone proteins. HMGB2 is member of the HMGB family, which plays fundamental roles in DNA replication, nucleosome assembly and transcription. HMGB2 is also a component of an ER-associated multiprotein complex (termed SET), which participates in the DNA-repair response to oxidative stress [50]. The up-regulation of HMGB2 transcript levels by LPS and copper provides the first evidence for the involvement of this DNA-bending protein in the Senegalese sole response to immunostimulants and oxidative agents. Finally, DDIT4L inhibits mammalian TOR-dependent functions, such as regulation of translation, mRNA turnover and transcription. Mammalian DDIT4L gene is known to be up-regulated at the transcriptional level in response to a variety of stresses, including pro-inflammatory stimuli and DNA damages [51]. In agreement with these studies, sole DDIT4L mRNA was up-regulated by both treatments. Our *in vivo* LPS induction was similar in extent and kinetic profile to that previously observed in isolated trout macrophages [52].

Overall these findings have demonstrated the utility of the microarray and qRT-PCR techniques to distinguish between the contrasting stress responses relating to exposure to the two agents. We provide profiles of gene expression changes that are in accord with the anticipated differential mechanisms of action and show promise as biomarkers in the monitoring of exposure and impact of both infection and its treatment, in relation to improved management and welfare of fish in aquaculture.

Acknowledgments

This work was supported by CICYE (00516), and co-financed by FEDER funds. I.O.-J. held a predoctoral fellowship (Junta de Andalucía). We thank B. Espejo-Reyes for technical support. Microarray development was funded by the UK NERC post-genomic and proteomic programme grant NE/C507661/1.

Appendix A. Supplementary material

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.fsi.2009.02.020.

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